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Functional Characterization of the Promoter for the Gene Encoding Human Eosinophil Peroxidase*

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The molecular basis for commitment of progenitors to the eosinophil lineage and mechanisms by which eosinophil-specific genes are expressed and regulated during differentiation is unknown. Expression of eosinophil peroxidase (EPO) is restricted to the eosinophil lineage. To understand the mechanisms involved in transcriptional regulation of EPO gene expression, we cloned the region of the EPO gene upstream of the transcriptional start site and analyzed the cis-acting elements required for EPO promoter activity in an eosinophil-inducible leukemic cell line, HL-60-C15. The 5′-flanking region of the EPO gene containing 1.5 kilobases of sequence upstream of the transcriptional start site was subcloned into the promoterless pXP2-luciferase vector. The EPO-pXP2 construct and 5′ deletion mutants were electroporated into HL-60-C15 cells and luciferase reporter activity assessed. The −1.5-kilobase EPO-pXP2 promoter construct reproducibly expressed >120-fold more luciferase activity than did promoterless pXP2, and a 12-fold (90%) decrease in promoter activity was obtained when sequences between −122 and −45 base pairs (bp) were deleted. The specificity of the EPO promoter for the eosinophil lineage was analyzed by transfecting the EPO-pXP2 constructs and deletion mutants into HL-60-C15 cells and the parental HL-60 line; EPO promoter activity was 8–10-fold less in the HL-60 parental line, suggesting lineage specific elements in the −122 to −45 bp region. To further characterize regulatory sequences important for promoter activity, we performed linker-scanning analysis on the −122 to −45 bp region and identified a number of positively and negatively acting elements in the promoter. DNase I footprinting was performed with HL-60-C15, HL-60, and HeLa nuclear extracts to identify nuclear proteins that may bind to the functional elements; these experiments identified three protected regions of the EPO promoter which correspond to the functional segments defined by linker-scanning analysis and which contain consensus, potential binding sites for Egr-1, H4TF-1, PuF, CTCF, UBP-1, and GaEII transcription factors. Further study of EPO promoter regulation should elucidate unique transcriptional features of eosinophil gene regulation in granulocyte development.

Eosinophil differentiation from precursor cells in the bone marrow is regulated by a number of different cytokines, including IL[1]-3, GM-CSF, and IL-5 (1–4). However, the molecular basis for the commitment of progenitors to the eosinophil lineage remains unknown. Augmented eosinophil differentiation in the bone marrow and accumulation of eosinophils in tissues and peripheral blood is associated with parasitic infections and allergic diseases, as well as certain malignancies and idiopathic syndromes including the hypereosinophilic syndrome (5–7). The large specific granule of the eosinophil contains four highly cationic proteins, including eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) (8), considered to play a seminal role as mediators of inflammation and tissue damage in the pathogenesis of these eosinophil-associated diseases (6, 9, 10). The biochemical and functional properties of these cationic proteins have been extensively investigated (9) and their cDNAs and genomic DNAs recently cloned and sequenced (11–21).

Human EPO is a heme-containing glycoprotein and a member of the peroxidase gene family that includes the closely related neutrophil myeloperoxidase as well as porcine and human thyroid peroxidases and human glutathione peroxidase (20, 21). EPO is a potent toxin for parasites, mammalian cells, and tissues both in the presence and absence of H_2O_2, and halide or thiocyanate cofactors (22–26), and may contribute to the pathogenesis of epithelial damage and bronchial hyperreactivity in human asthma (25), and cardiac damage in the eosinophilic endomyocardial fibrosis associated with the hypereosinophilic syndrome (27). EPO induces histamine release from rat mast cells (28), inactivates leukotrienes (29), and is cytotoxic for the microfilarial stage of *Brugia pahangi* (30). As purified from the large specific granule of the eosinophil, EPO is a heterodimer consisting of a heavy chain of 50–55 kDa and a light chain of 14–15 kDa (31); both chains are encoded by a single mRNA species in a manner similar to that of myeloperoxidase (21, 32). The similarity of the EPO nucleotide sequence to that of other eukaryotic peroxidases suggests that these peroxidases comprise a multigene family that evolved by gene duplication (20, 21). Like the gene for myeloperoxidase (33), the EPO gene consists of 12 exons and 11 introns spanning approxi-

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[1] The abbreviations used are: IL, interleukin; EPO, eosinophil peroxidase; MBP, major basic protein; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; CLC, Charcot-Leyden crystal protein; RLU, relative light units; bp, base pair(s); PCR, polymerase chain reaction; hGH, human growth hormone; GM-CSF, granulocyte-macrophage colony-stimulating factor; kb, kilobase(s); CMV, cytomegalovirus.

mately 12 kb of DNA (20). Expression of EPO mRNA has thus far been demonstrated in eosinophil-committed sublines of HL-60 (HL-60 3+C-5 (20) and HL-60-C15),[2] but not in a neutrophilic HL-60 subline (14-C) or parental HL-60 cells (20). EPO mRNA expression is also strongly up-regulated in umbilical cord blood progenitors induced to differentiate into eosinophils with a combination of IL-3, IL-5, and GM-CSF (34) or bone marrow progenitors induced with IL-5 alone,[2] with decreased expression during eosinophil maturation (34). Thus, expression of the EPO gene at the RNA level has been detected exclusively in the eosinophil lineage and not in other myeloid hematopoietic lineages.

To identify transcription factors involved in regulating the commitment and differentiation of hematopoietic progenitors toward the eosinophil lineage, we have analyzed the cis-acting elements and DNA-binding proteins that may control EPO gene expression. Using an eosinophil-inducible cell line, HL-60-C15, derived from the HL-60 promyelocytic leukemia line (35, 36), we have identified the minimal upstream region of the EPO gene required for promoter activity and localized a number of positively and negatively acting promoter elements in this region that may participate in regulating the expression of EPO during normal eosinophil development.

EXPERIMENTAL PROCEDURES

Cell Culture—The eosinophil-committed subline of the HL-60 promyelocytic leukemia cell line, HL-60-C15 (36) (American Type Culture Collection (ATCC), Rockville, MD; ATCC CRL 1964, a gift of Dr. Steven Fischkoff), was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine (Life Technologies, Inc.) and passaged twice weekly (37). HL-60-C15 cells (2×10^5/ml) were induced with 0.5 mM butyric acid (Sigma) for 48 h as previously described (36). For mRNA stability studies, actinomycin D (Pharmacia, Uppsala, Sweden) in ethanol was added to a final concentration of 10 μg/ml for the indicated times to uninduced HL-60-C15 cells and to HL-60-C15 cells previously induced for 48 h with 0.5 mM butyrate. Other myeloid and non-myeloid cell lines utilized in these studies including the promyelocytic HL-60 line (ATCC CCL 240), monocytic U937 line (ATCC, CRL 1593), BJA/B B lymphocytic cells (38), Jurkat T lymphocytic cells, and the cervical carcinoma line, HeLa (ATCC CCL 2) were maintained by passage twice weekly in Iscove's modified Dulbecco's medium (HL-60, U937, BJA/B, and Jurkat) or Dulbecco's modified Eagle's medium (HeLa) supplemented with 10% fetal bovine serum and 2 mM L-glutamine.

Northern Blot Analysis—Total RNA was prepared from uninduced and butyrate-induced HL-60-C15 cells by the guanidium isothiocyanate method (39). The RNA (15–20 μg/lane) was denatured in formamide-formaldehyde followed by electrophoresis in 1% agarose-formaldehyde gels. The RNA was then transferred to Hybond-N nylon membranes (Amersham Corp.) and the blots probed sequentially with exon 11 of EPO genomic DNA (the exon with the least similarity to myeloperoxidase) and a 28 S RNA oligonucleotide probe (5'-AACGATCAGAG-TAGTGG-3') for internal control of RNA loading. Hybridization with the random-primed DNA probe for EPO was performed at 43 °C in 50% formamide, 6 × SSC, 0.2% Ficoll polyvinylpyrrolidone, and 0.1% sodium dodecyl sulfate (SDS). Filters were washed twice in 2 × SSC with 0.2% SDS at 53 °C for 30 min and twice in 0.2% SSC with 0.2% SDS at 55 °C for 30 min. Autoradiography was performed at −80 °C with Kodak XAR-5 film. The filter was then stripped and rehybridized overnight to a 5' end-labeled 28 S RNA oligonucleotide probe in 6 × SSPE, 0.1% SDS, 5 × Denhardt's solution, and 80 μg/ml of denatured sheared salmon sperm DNA at 44 °C, washed in 6 × SSPE and 0.1% SDS twice at 20 °C, and washed once at 44 °C for 15 min. Autoradiograms of Northern blots and run-on assays (see below) were quantitated by scanning on a ScanJet Plus Scanner (Hewlett-Packard) followed by densitometry analysis using ImageQuant™ Software (Molecular Dynamics).

Nuclear Run-on Transcription Assay—Nuclear run-on assays were performed as previously described (40). The following DNAs were used to prepare slot-blots: a 0.6-kb *PstI/Bam*HI fragment containing hEPO exon 11 DNA in pBR322 (20), a 1.9-kb *Eco*RI/*Sal*I fragment containing the human 18 S RNA cDNA in pBR322 (kindly provided by Dr. Howard Young), and pBR322 vector alone. Autoradiograms were exposed at −80 °C with an intensifying screen.

Plasmids for Transient Transfections—The promoterless luciferase plasmid pXP2 was used for all promoter studies (41). A cytomegalovirus-human growth hormone (CMV-hGH) plasmid, for use as an internal control in transfections, was provided by Dr. Leonard Zon (Children's Hospital Medical Center, Boston, MA). A 1.5-kb *Eco*RI/*Bam*HI human EPO genomic fragment was filled in with Klenow enzyme and subcloned into the *Sma*I site of the pXP2 promoterless luciferase plasmid. The resulting construct contained approximately 1.5 kb of 5'-flanking DNA and extended 3' to +61 bp, where +1 denotes the previously identified transcriptional start site (20). Unidirectional deletions of the 1.5-kb/EPO-luciferase construct were prepared using Exonuclease III as previously described (42). Dideoxy sequencing using Sequenase (United States Biochemical Corp., Cleveland OH) was performed to identify the positions of the Exonuclease III end points (43).

Linker Scanning Analysis—A 10-bp linker containing an *Eco*RI restriction site (sequence 5'-CGGAATTCCG-3') was substituted for the wild type sequence of the −122-bp EPO promoter deletion construct between bp −122 and −113 (−122/−113), bp −112 and −103 (−112/−103), bp −102 and −93 (−102/−93), bp −92 and −83 (−92/−83), bp −82 and −73 (−82/−73), bp −72 and −63 (−72/−63), bp −62 and −53 (−62/−53), and bp −52 and −43 (−52/−43) using oligonucleotide directed PCR mutagenesis (44). The PCR fragments were cloned into the luciferase vector pXP2 and resulting constructs sequenced as above to confirm the correct placement of the linker oligonucleotide.

Transient Transfections—DNA was prepared and transfected as previously described using 1.5×10^7 cells/transfection (37, 45) with minor modifications. Briefly, the HL-60-C15 subline and parental HL-60 cell lines were electroporated at 280 volts, 960 μF, and the U937, BJA/B, and Jurkat lines at 300, 280, and 250 volts, 960 μF, respectively, conditions previously optimized for these lines (37, 45–47). Luciferase activity in cell lysates prepared 4–6 h post-transfection was measured as relative light units (RLU) using a monolight 2010 Analytical Luminescence Laboratory luminometer (San Diego, CA) as previously described (37, 45); cell extracts were prepared in 500 μl of 1% Triton X-100, 25 mM Gly-gly, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol, and 100 μl of the extract (equivalent to $\sim 3 \times 10^6$ cells) was analyzed for luciferase activity. Each transfection contained DNA equivalent to 20 μg of the EPO-pXP2 construct and 0.5 μg of the CMV-hGH internal plasmid control. In some experiments, 25 μg of carrier DNA (pBluescriptII KS$^-$) was included, and sample DNA was reduced to 10 μg. The CMV-hGH co-transfection provided for standardization among the different myeloid and non-myeloid lines, different plasmid DNA preparations, and individual transfection experiments (47) and the carrier DNA decreased variability due to differences in the DNA preparations for the individual EPO promoter-pXP2 constructs (48). Growth hormone production was measured by assaying the culture supernatants of transfected cells with a commercially available radioimmunoassay kit (Nichols Institute Diagnostics, San Juan Capistrano, CA). The RLU from individual transfections were normalized for transfection efficiency based on the number of nanograms of growth hormone produced and are indicated as either RLU/ng/ml hGH or corrected RLU. An enhancer-containing vector, CMV-pXP2, was used as a positive control for each transfection as well. Individual transfection experiments were repeated at least three times, and the results are reported as mean relative light units/nanogram human growth hormone/milliliter (± S.E.).

Nuclear Extracts and DNase I Footprinting—Nuclear extracts for DNase I footprinting were prepared from 1×10^9 HL-60-C15, HL-60, and HeLa cells using the procedure described by Zhang *et al.* (49). The extraction buffer contained 2 μg/ml each of the protease inhibitors aprotinin, pepstatin A, trypsin inhibitor, leupeptin, and antipain. Approximately 15 mg of total nuclear protein were obtained from each cell line. The probe for DNase I footprinting, a ~350-bp DNA fragment extending from −289 to +61bp of the EPO promoter, was prepared by digesting the pXP2-EPO(M3) deletion mutant with *Hin*dIII and *Xho*I to release the promoter fragment and end-labeled with [^{32}P]dTTP and [^{32}P]dATP (49). DNase I footprinting was performed as previously described (49). Briefly, nuclear proteins (60 or 120 μg each) extracted from HL-60-C15, HL-60, or HeLa cells were incubated with 4 μg of double-stranded poly(dI·dC) and the gel-purified radiolabeled EPO probe in a total of 20 μl binding buffer (10 mM HEPES, pH 7.9, 30 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride, 12% glycerol); binding reactions were allowed to proceed for 60 min on ice prior to the addition of DNase I.

Sequence Analysis—Putative regulatory elements (consensus sequences) within the functionally active regions of the EPO promoter

[2] Y. Yamaguchi, E. A. Albee, H. N. Steinberg, S. Nagata, L.I. Zon, D. G. Tenen, and S. J. Ackerman, manuscript in preparation.

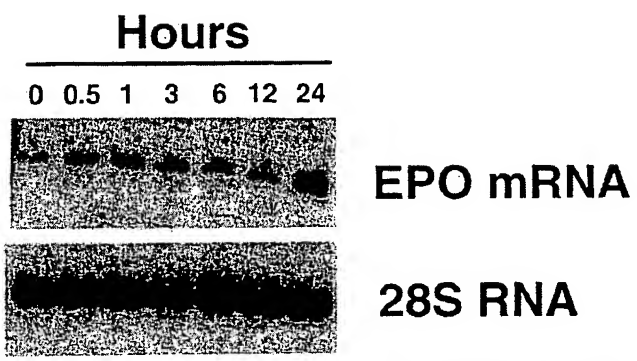


FIG. 1. **Northern blot analysis for mRNA encoding EPO in HL-60-C15 cells induced with butyrate.** HL-60-C15 cells were cultured in the presence of butyrate (0.5 mM) for 24 h. Total RNA was obtained at 0, 0.5, 1, 3, 6, 12, and 24 h of culture. Each lane contained 15 μg of total RNA. The blot was sequentially probed with the EPO cDNA and a 28 S RNA-specific oligonucleotide probe as a control for equivalent RNA loading.

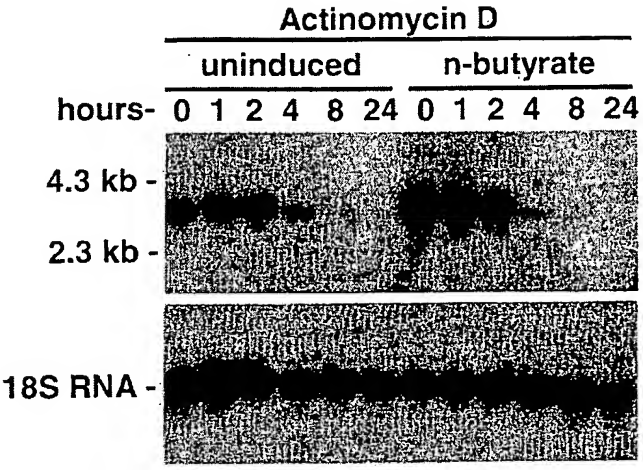


FIG. 2. **Half-life of EPO mRNA in HL-60-C15 cells.** Northern blot of total RNA (15 μg/lane) of HL-60-C15 cells exposed for 0–24 h to actinomycin D (10 μg/ml). *Lanes 1–6*, uninduced HL-60-C15 cells; *lanes 7–12*, HL-60-C15 cells induced with 0.5 mM butyric acid for 48 h prior to the addition of actinomycin D. *Lanes 1* and *7*, no addition of actinomycin D; *lanes 2–6* and *8–12*, treatment with actinomycin D for 1, 2, 4, 8, and 24 h, respectively.

and in the 5'-flanking regions of the MBP, ECP, EDN, and myeloperoxidase genes were identified by searching the Ghosh transcription factors data base (50).

RESULTS

Expression of EPO mRNA Is Transcriptionally Up-regulated during Butyrate Induction of HL-60-C15 Cells—It has previously been shown that butyric acid treatment induces granulogenesis in HL-60-C15 cells (36, 51), markedly up-regulates the expression of mRNAs encoding the major eosinophil granule-associated proteins, including CLC, ECP, EDN, EPO, and MBP by 1–3 days[2] (37, 51), and stops continued proliferation of the line after 5–7 days of culture.[3] In order to determine the effect of shorter term exposures of butyrate on EPO mRNA expression, Northern blot analysis was performed on total RNA isolated from HL-60-C15 cells cultured with butyrate for up to 24 h. As shown in Fig. 1, butyrate induction of HL-60-C15 up-regulated the expression of EPO mRNA with a 3.7-fold increase in mRNA accumulation by 24 h. To determine whether the increased accumulation of EPO mRNA was mediated at the transcriptional and/or post-transcriptional level, message stability was analyzed in experiments in which actinomycin D, an RNA synthesis inhibitor, was added for 0–24 h to both uninduced HL-60-C15 cells and to cells previously induced with butyrate for 48 h (Fig. 2). As shown in Fig. 2, the half-life of the EPO mRNA in both the uninduced and butyrate induced HL-60-C15 cells treated with actinomycin D was 2.5 h, suggesting that the accumulation of EPO mRNA in butyrate-induced HL-60-C15 cells was mediated at the transcriptional level and not due to increased message stability. To further confirm this result, nuclear run-on assays were performed using HL-60-C15 cells, both uninduced and induced for 48 h with butyrate; Northern blot analysis for EPO mRNA expression was performed on total RNA prepared from the same cells. As shown in Fig. 3*A*, scanning densitometry indicated that butyrate induced a 2-fold increase in the EPO transcriptional rate over that of the uninduced cells and scanning densitometry of Northern blots for EPO mRNA likewise demonstrated a comparable 2-fold increase in the same butyrate-induced HL-60-C15 cells (Fig. 3*B*). These data suggest that expression of the EPO gene is transcriptionally up-regulated during butyrate induction of the HL-60-C15 subline.

Deletion Analysis of the EPO Promoter Demonstrates the Presence of Negative and Positive Regulatory Elements—In order to locate the cis-acting element(s) required for EPO promoter activity, we analyzed luciferase activity in HL-60-C15 cells transiently transfected with various EPO promoter constructs subcloned into the promoterless pXP2 luciferase expression vector. An EPO genomic fragment containing 1.5 kb of sequence upstream of the transcriptional start site (20) was subcloned into the promoterless pXP2 luciferase vector as outlined in Fig. 4. When transiently transfected into uninduced HL-60-C15 cells, the −1.5-kb/EPO-luc promoter construct reproducibly expressed >120-fold more luciferase activity than did the promoterless pXP2 control. To localize regulatory elements in the EPO promoter, a series of seven deletion mutants (M1, M2, M2.3, and M3-M6) in the −1.5-kb/EPO-luc construct were produced using convenient restriction sites and exonuclease III. In butyrate-induced HL-60-C15 cells, promoter activity of these constructs was consistently 2–3-fold greater than in the uninduced cells (Fig. 5*A*). This finding was consistent with the 2-fold increase in steady state mRNA levels from Northern blot analysis and the 2-fold increase in the transcriptional rate in nuclear run-on transcription assays. In uninduced cells, no significant alterations in promoter activity were observed when sequences between −1.5 kb and −289 bp were deleted (Fig. 5*A*), although deletion of the region between −289 and −122 bp resulted in a 2-fold increase in activity and a loss of the butyrate inducibility of the promoter. Further deletions to −65 and −45 bp produced more than a 4- and 12-fold (90%) decrease in luciferase activity, respectively, when compared with the −122-bp construct (Fig. 5*B*).

Lineage and Myeloid Specificity of the EPO Promoter—The specificity of the EPO promoter for the eosinophil lineage was assessed by transfecting the wild type and various deletion constructs into both HL-60-C15 cells and the HL-60 parental cell line (Fig. 6). The activity of the EPO promoter was approximately 8.5-fold lower in the parental HL-60 line than in the eosinophil-committed HL-60-C15 subline (Fig. 6). Further, the EPO-pXP2 promoter constructs were likewise considerably less active in HL-60 cells induced toward neutrophilic differentiation with Me_2SO (Fig. 6). However, in other myeloid (U937 myelomonocytic cells) and non-myeloid leukemic lines (BJA/B B lymphocytic cells, Jurkat T lymphocytic cells), the activity of the EPO promoter and the mutant constructs was comparable to or greater than that obtained in the eosinophilic HL-60-C15 line (data not shown).

[3] Y. Yamaguchi, D. G. Tenen, and S. J. Ackerman, unpublished observations.

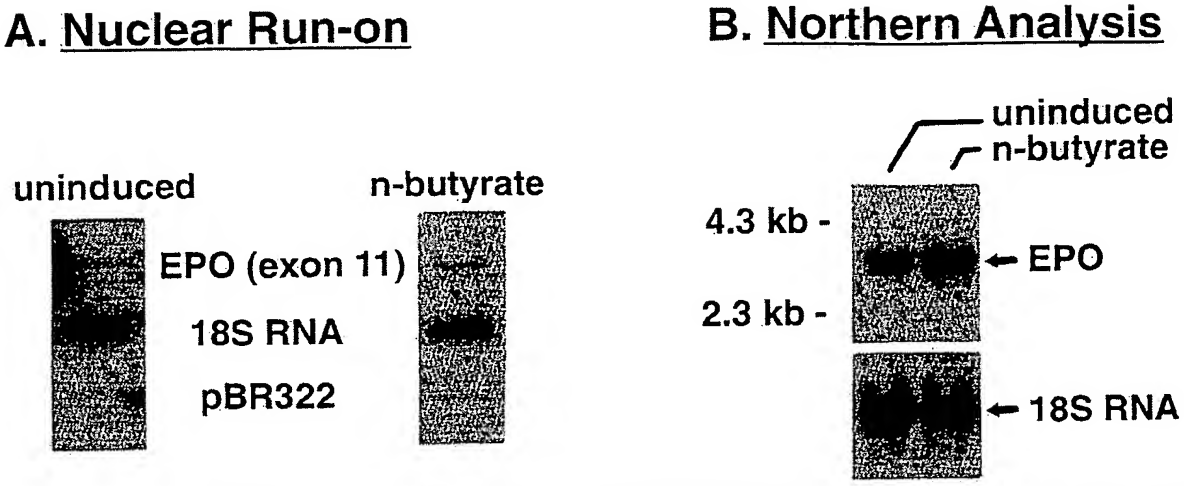


FIG. 3. **Nuclear run-on and Northern analysis of HL-60-C15 cells induced with butyrate.** *Panel A*, nuclear run-on transcription analysis was performed with nuclei isolated from uninduced and 48 h butyrate-induced HL-60-C15 cells. Equal numbers of counts were hybridized to duplicated filters containing a molar excess of the indicated probes. *Panel B*, Northern blot analysis of RNA from an aliquot of the same HL-60-C15 cells used for the nuclear run-on assay. *Lane 1*, 15 μg of total RNA from uninduced HL-60-C15 cells. *Lane 2*, 15 μg of total RNA from HL-60-C15 cells induced with butyrate for 48 h. The blot was probed sequentially with an EPO (exon 11) probe, followed by a cDNA probe for 18 S RNA as a control for equivalent RNA loading.

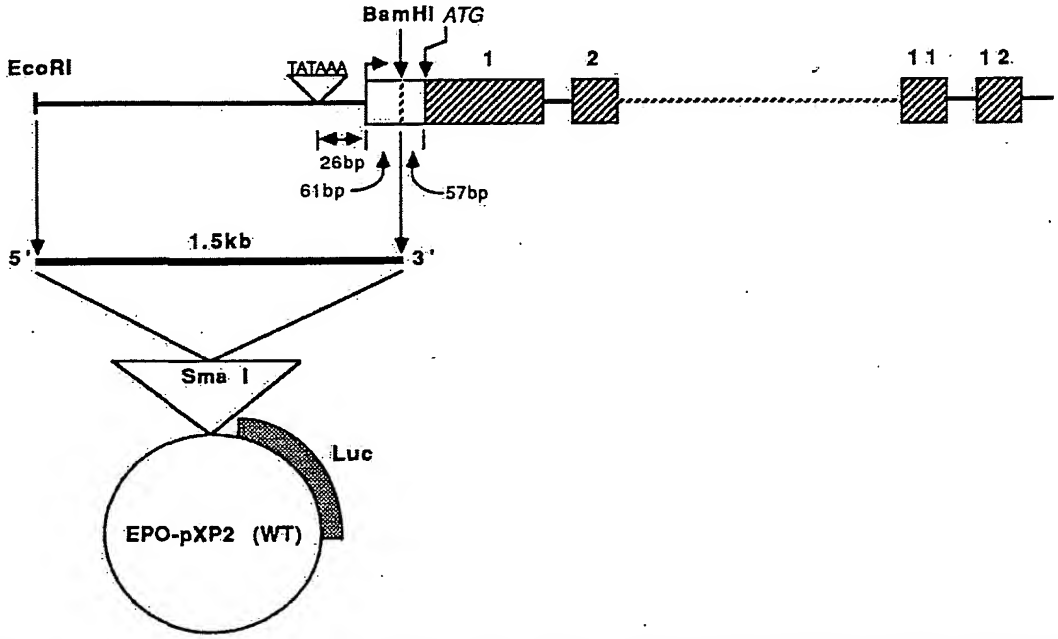


FIG. 4. **Structure of the human EPO genomic fragment subcloned into the promoterless pXP2 luciferase vector.** A 1.5-kb *Eco*RI/*Bam*HI fragment of the 5'-flanking region of the human EPO gene (20), including the transcriptional start site and 61 bp of the 5'-untranslated region, was subcloned into the *Sma*I site of the promoterless pXP2 luciferase plasmid.

Negative and Positive Regulatory Elements Map within the −112/−93 and −62/−43 Regions of the EPO Promoter—To more precisely locate the functional sequences required for EPO promoter activity in the region between the transcriptional start site and −122 bp, eight linker-scanning mutants were constructed by inserting a 10-bp oligonucleotide consecutively every 10 bp in place of the wild type promoter sequence (Fig. 7*A*); this resulted in a 60–100% change in the wild type sequence in each 10 bp region as indicated in Fig. 7*B*. A 3-fold increase in EPO promoter activity relative to either the wild type or the M4 deletion mutant was seen when the sequence between bp −112/−103 was replaced by the linker, whereas a 2-fold decrease was obtained for the −102/−83 bp replacement and a 3-fold decrease in activity resulted when sequences between bp −62 and −53 or bp −52 and −43 were replaced (Fig. 7*A*).

DNase I Footprinting Identifies Protected Regions of the EPO Promoter Corresponding to the Functionally Active Elements Identified by Linker-scanning Analysis—To determine whether any nuclear proteins bind to the cis-acting elements in the EPO promoter regions identified in the above functional analysis, DNase I footprinting was performed using a 350-bp upstream fragment including the transcriptional start site (−289 to +61bp) and nuclear protein extracts prepared from the uninduced HL-60-C15 line, the parental HL-60 line and HeLa cells (Fig. 8). Three protected regions (*I-III*) were identified corresponding to the sequences from −71/−42 (region I), −111/−88 (region II), and −155/−130 (region III). Footprinted regions I and II correspond to the functional sequences of the promoter identified in the linker-scanning analysis as containing both positively (−71/−42, −102/−93) and negatively (−112/−103) act-

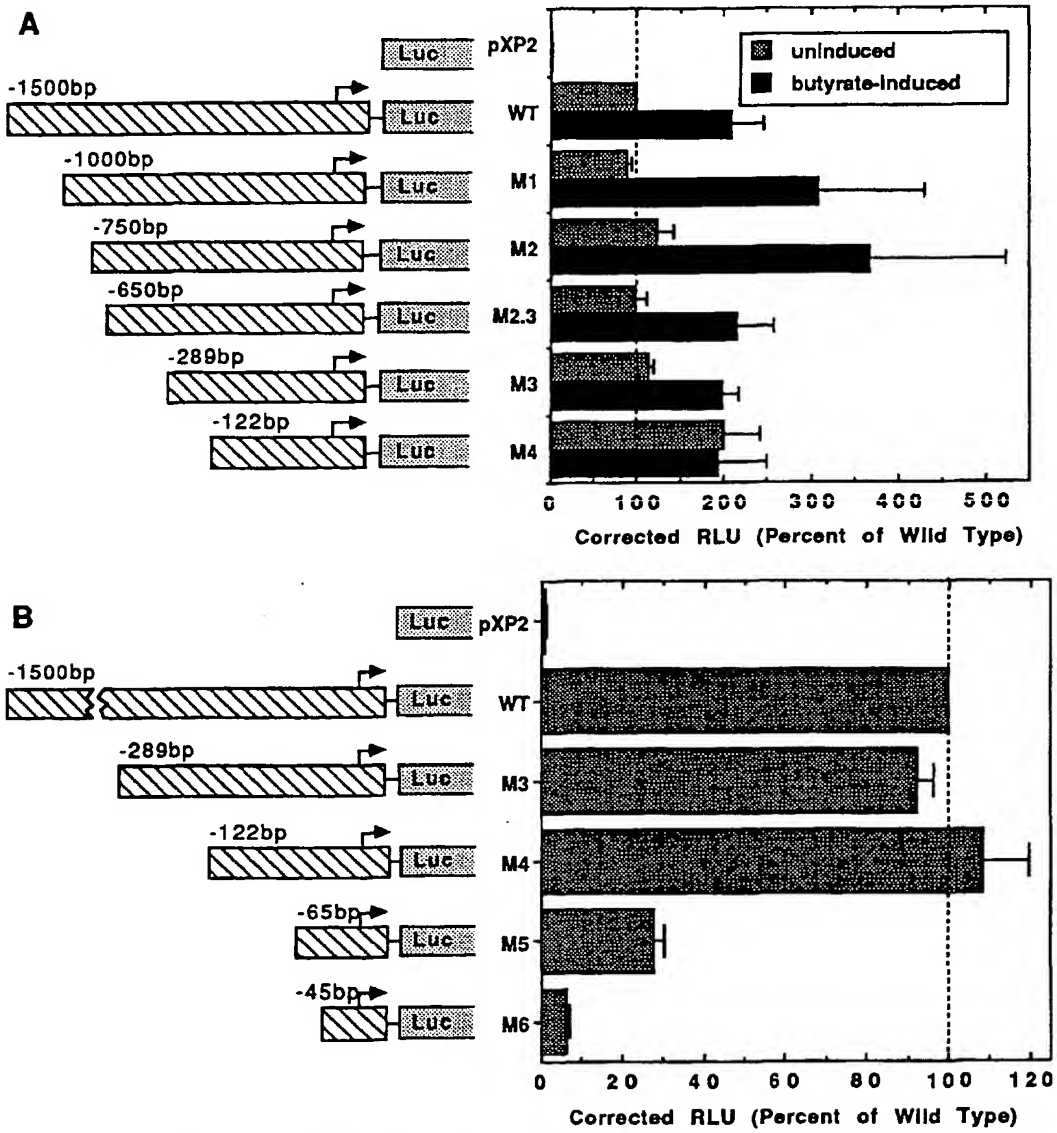


FIG. 5. **Functional activity of the EPO-pXP2 luciferase constructs in HL-60-C15 cells.** *Panel A*, schematic representations of the EPO-pXP2 luciferase constructs are shown on the *left*. Twenty μg of each pXP2-EPO construct were transfected by electroporation into both uninduced and butyrate-induced HL-60-C15 cells. One-fifth of the transfected cells was assayed for luciferase activity after culture for 4.5 h. RLU have been corrected based upon the amount (ng/ml) of human growth hormone released into the culture supernatants for each individual transfection. Luciferase activities in each construct are shown as the percent activity, relative to the mean activity of the longest, 1.5-kb wild type (*WT*) EPO-pXP2 promoter construct. The mean ± S.E. for three replicate experiments is shown. *Panel B*, promoter activities of additional 5′ deletion mutants of the EPO-pXP2 construct in uninduced HL-60-C15 cells. Twenty-five μg of pBluescriptII KS⁻ was transfected as carrier DNA with 10 μg of each EPO-pXP2 construct into HL-60-C15 cells. Luciferase activities have been normalized for the amount of hGH produced by the co-transfected control CMV-hGH plasmid. Corrected RLU for each construct are shown as the percent activity relative to the mean activity of the longest 1.5-kb EPO promoter construct. The mean ± S.E. for three replicate experiments is shown.

ing elements, respectively. Region III corresponds to a potential negative-acting element as defined by the activity of deletion mutants M3 *versus* M4 (Fig. 5*A*), a region that may participate in the butyrate inducibility of EPO promoter activity (M3 *versus* M4, Fig. 5*A*).

Functional Cis-elements and Potential Trans-acting Factors for EPO Gene Regulation—As shown in Fig. 9, the 306 bp of published DNA sequence (20) upstream from the transcriptional start site were evaluated for potential regulatory sequences by comparison to the Ghosh transcription factors database (50). Consensus sequences were identified in the upstream 306 bp that represent potential binding sites for a number of known transcription factors (Fig. 9*A*) and that correspond to the functionally active regions of the promoter as defined by both DNase I footprinting and linker-scanning analyses (Fig. 9*B*). Region I, defined by DNase I footprinting (Fig. 8) and functionally active elements (Fig. 7), contains potential binding sites for four different factors including PuF (52, 53), Egr-1 (54, 55), H4TF-1 (54–57), and CTCF (58). Region II, which covers both positively and negatively acting elements, contains a potential binding site for the UBP-1 factor (59, 60). Region III, which covers sequences that may contain a weak negatively acting element, contains a second consensus sequence for PuF and as well as a potential GaEII site (61). Finally, there is a putative Sp1-binding site (62, 63) at −75 to −83 (Fig. 9*A*), albeit in a region of the promoter that showed no functionally important sequences in the linker scanning analysis (Fig. 7*A*, mutant M4–5) and did not show a footprint using

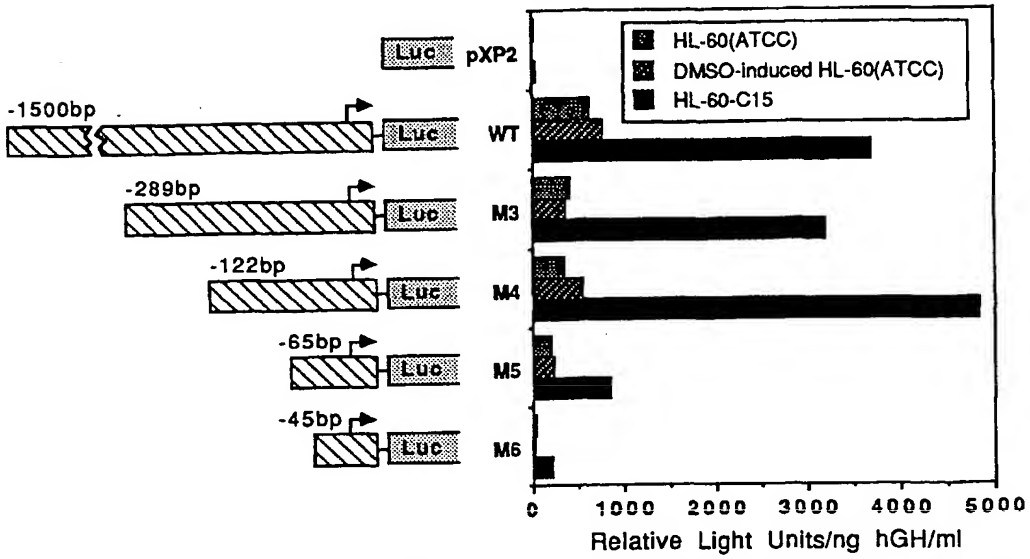


FIG. 6. **Comparative promoter activity of the EPO-pXP2 luciferase constructs in HL-60-C15, HL-60, and Me_2SO-induced HL-60 cells.** Ten μg of each EPO-pXP2 luciferase construct was transiently transfected and expressed in uninduced HL-60-C15 cells and in the parental HL-60 line with or without prior treatment for 40 h with Me_2SO (1.1% v/v) to induced neutrophilic differentiation. Luciferase activities have been normalized for growth hormone production from the CMV-hGH co-transfected control plasmid.

nuclear extracts from HL-60 and HeLa previously shown to identify an Sp1 site in the CD14 (64) and CD11b promoters (65).

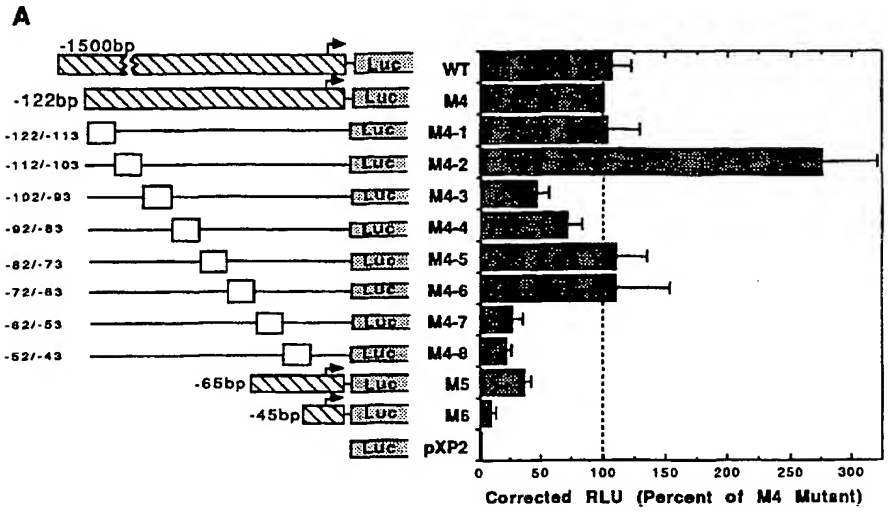
DISCUSSION

In this paper, we report the identification and functional characterization of the promoter for the human eosinophil peroxidase gene, a gene expressed exclusively in the differentiation of myeloid progenitors to the eosinophil lineage. In contrast to the gene for myeloperoxidase (33), which has relatively weak promoter activity *in vitro* and requires the use of a heterologous promoter for functional analysis (66, 67), the EPO gene has a strong promoter whose principal regulatory elements are located within the first ~100 base pairs upstream of the transcriptional start site. Study of this eosinophil promoter should provide an opportunity to assess the lineage and differentiation specific cis-acting elements and trans-acting factors involved in the expression of eosinophil-specific genes during the program of eosinophil development.

Activity of the EPO promoter was analyzed in the eosinophil-inducible HL-60-C15 cell line which can undergo either spontaneous or alkaline pH and/or butyric acid induced differentiation into highly granulated cells in culture (35, 36, 68, 69). Approximately 5–20% of uninduced HL-60-C15 cells are granulated during routine passage and as such they constitutively express EPO (35) as well as the genes encoding all the other eosinophil granule-associated proteins.[2] Culture of HL-60-C15 with butyric acid rapidly up-regulates the expression within 24 h of the genes encoding ECP, EDN[2] (51), MBP,[2] and CLC protein (37). In the present analyses, butyrate induction of eosinophilic differentiation of HL-60-C15 produced a 2-fold increase in the EPO transcriptional rate, a finding that was consistent with a 2-fold increase in mRNA levels and that could not be accounted for by an increase in message stability in this line. Likewise, butyrate induction induced a 2–3-fold increase in the functional activity of the EPO-pXP2 promoter constructs. The lack of a more marked increase in the EPO transcription rate in HL-60-C15 cells induced with butyrate likely reflects the finding that this subline is already partially differentiated such that the EPO gene is already being transcribed. In comparison, the gene for myeloperoxidase is constitutively expressed in HL-60 cells and both transcription and steady state mRNA levels decrease upon induction toward neutrophilic differentiation with either Me_2SO (70, 71) or retinoic acid (33, 46, 72).

Transient transfections with the EPO promoter deletion constructs suggested a butyrate-responsive element located within the 1.5 kb of sequence upstream of the EPO transcriptional start site (Fig. 5*A*). Riggs *et al.* (73) have shown that the addition of butyrate leads to hyperacetylation of histone H4 in HeLa and Friend erythroleukemia cells. More recently, Klehr and colleagues (74) have reported that butyrate appears to exert its action at the level of chromatin structure acting synergistically with so-called scaffold-attached regions. Treatment of HL-60-C15 cells with butyrate may also induce hyperacetylation of nucleosomal histone and alter chromatin conformation to increase the transcriptional rate of EPO or other eosinophil genes. Alternatively, the general butyrate inducibility of gene expression in the HL-60-C15 line may be independent of or unrelated to the specific transcriptional events that are important for the differentiation of this leukemic cell line to the eosinophil lineage.

The expression of two other eosinophil granule protein genes, including CLC (37) and MBP,[3] was also transcriptionally up-regulated during butyrate-induced eosinophil differentiation of the HL-60-C15 subline. Our results are consistent with those of Gruart and colleagues (34) who have suggested, based only mRNA stability experiments, that expression of the genes encoding all the eosinophil granule cationic proteins (EPO, MBP, EDN, and ECP) is regulated primarily at the transcriptional level during eosinophil differentiation of umbilical cord blood-derived progenitors. Of interest, the half-life of EPO mRNA was reported to be more than 12 h in umbilical cord blood mononuclear cells induced toward eosinophil differentiation with a combination of IL-3, GM-CSF, and IL-5 (34). In contrast, the half-life of EPO mRNA in the present analyses was only 2.5 h in both uninduced or butyrate-induced HL-60-C15 cells. One potential explanation for this marked difference in mRNA half-life may be the more rapid cell cycle of the HL-60-C15 leukemic cell line. Alternatively, induction with cytokines such as IL-3, GM-CSF, or IL-5 may serve to stabilize the EPO mRNA in the developing cord blood-derived eosinophil progenitors.



B

			Sequence	% Change
EPO	-122/-113	Wild Type	CCCATGAAAA	
	-122/-113	Linker Scan	CGGAATTCCG	80
EPO	-112/-103	Wild Type	CCAGAGAGAA	
	-112/-103	Linker Scan	CGGAATTCCG	80
EPO	-102/-93	Wild Type	GTTTCAGAAC	
	-102/-93	Linker Scan	CGGAATTCCG	100
EPO	-92/-83	Wild Type	TCCACCCAAG	
	-92/-83	Linker Scan	CGGAATTCCG	80
EPO	-82/-73	Wild Type	AGGCTGGGTT	
	-82/-73	Linker Scan	CGGAATTCCG	80
EPO	-72/-63	Wild Type	TCTAGGGCCC	
	-72/-63	Linker Scan	CGGAATTCCG	70
EPO	-62/-53	Wild Type	AGAGCTGCCC	
	-62/-53	Linker Scan	CGGAATTCCG	60
EPO	-52/-43	Wild Type	TCCCCCACCC	
	-52/-43	Linker Scan	CGGAATTCCG	80

FIG. 7. **Linker-scanning analysis of the minimum 122-bp EPO promoter in uninduced HL-60-C15 cells.** *Panel A*, comparative promoter activity for transient transfections of HL-60-C15 cells with the wild type, M4 mutant, and eight linker scanning mutants of the EPO promoter in the pXP2-luciferase vector. The *schematic* on the *left* shows wild type sequences as *lines* or *diagonally striped rectangular boxes*, and the 10-bp sequences replaced by the linker oligonucleotide by the *square* (*open*) *boxes*. Three independent transfection experiments were performed; the average activities (±S.E.) relative to the activity of the M4 mutant (100%) are shown. Luciferase activity, measured as RLU, was first normalized for transfection efficiency (corrected RLU) as described under "Experimental Procedures" prior to calculating the averages and percentage activity relative to the M4 deletion mutant of the EPO promoter. For reference, the mean activities (±S.E.) of the wild type −1.5 kb/luc and M4 (−122 bp/luc) mutant constructs were 8,212 ± 2,958 and 7,448 ± 1,803 RLU/ng/ml hGH, respectively, and that of the promoterless pXP2 control was 62 ± 18 RLU/ng/ml hGH. *Panel B*, the wild type and sequence replacements for the individual linker-scanning mutations in *panel A* are shown. The percent change from the wild type sequence is indicated on the *right*.

As summarized in Fig. 9*A*, sequences between bp −122 and −45 of the EPO promoter contained essentially all the necessary sequence elements required for maximal functional activity in HL-60-C15 cells as well as other myeloid and non-myeloid lines. The observation that the EPO promoter constructs were likewise active in the myelomonocytic U937 line and in T and B lymphocytic lines (Jurkat and BJA/B, respectively) suggests a lack of myeloid- or eosinophil-specific elements in the 1.5 kb of upstream sequence analyzed thus far. However, it is entirely possible that tissue-specific negative or positive regulatory elements are located further upstream or downstream in the EPO gene. For example, Burn and colleagues (47) have identified a 3′ enhancer in the gene for the human CD34 stem cell antigen that increases promoter activity in a cell type-specific manner. Alternatively, the EPO gene may be similar to that of myeloperoxidase, which has recently been shown to have enhancers with estrogen and retinoic acid-like response elements within introns 7 and 9 and which are functional in myeloperoxidase expressing HL-60 and SKM-1 cells lines (67). Of note, all EPO promoter constructs, including those with sequences up to 1.5 kb upstream of the transcriptional start site, were 8–10-fold less active in the parental HL-60 line than in the eosinophilic HL-60-C15 subline (Fig. 6), suggesting some lineage specificity when analyzed within this promyelocytic leukemia line. The EPO promoter constructs were likewise significantly less active in the parental HL-60 line induced toward neutrophilic differentiation with Me_2SO. Taken together, these observations suggest that there may be silencer elements which normally suppress promoter activity of the sequence between bp −122 and −45 in other myeloid or non-myeloid lineages, elements not present within the 1.5 kb of upstream sequence analyzed thus far. Silencer elements that act to suppress enhancer function in a lineage-specific manner have been described for the T cell receptor α enhancer in $\alpha\beta$ *versus* $\gamma\delta$ T cells (75).

Functional analyses of linker-scanning mutants in the bp −122 to −45 region of the EPO promoter have identified a number of positively and negatively acting elements that could be important in regulating the expression of the EPO gene in particular and eosinophil granule protein genes in general. The basal EPO promoter may be controlled by both negatively and positively acting cis-elements in bp −112 to −103 and bp −62 to −43 segments of the gene, respectively. DNase I footprinting of the EPO promoter identified three protected regions upstream of the TATA box corresponding to the functionally active elements that were defined by the linker-scanning analysis. These

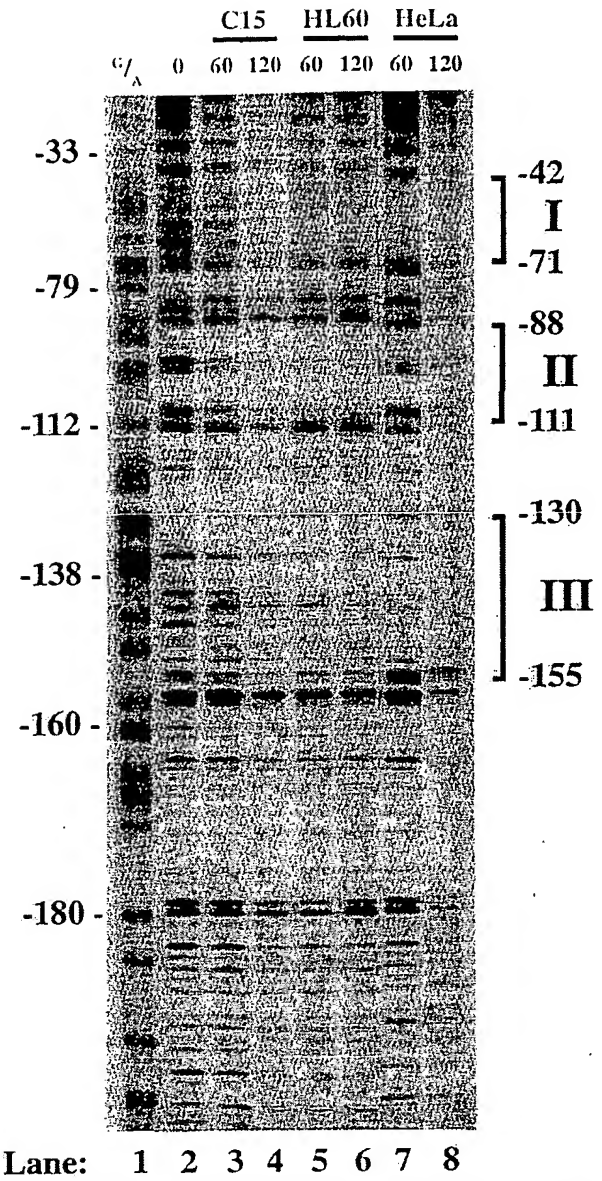


FIG. 8. DNase I protection of the EPO promoter by nuclear proteins from HL-60-C15, HL-60, and HeLa cell lines. A ^{32}P end-labeled 350-base pair fragment of the EPO promoter extending from −289 to +61bp was incubated with 0, 60, or 120 μg of nuclear proteins prepared from HL-60-C15, parental HL-60, or HeLa cells as indicated at the top of *lanes 2–8*. *Lane 1* (G/A) is a sequencing marker consisting of the DNA probe subjected to guanosine/adenine-specific Maxam-Gilbert cleavage (80) used to identify the number of nucleotides upstream of the transcription initiation site (*numbers* at the *left side* of the panel). The footprinted regions of sequence protected from DNase I digestion are *bracketed* and the *Roman numerals* (*I-III*) refer to the entire protected region.

coincident regions contain potential binding sites (*i.e.* consensus sequences) for a number of known transcription factors including Egr-1, H4TF-1, PuF, CTCF, UBP-1, and GaEII (Fig. 9). Egr-1, also know as Zif268, NGF-1A, and Krox24 (55), is a zinc finger-containing transcription factor that binds to a CTCCCCCAC consensus sequence. Egr-1 is an immediate early response gene that is induced by various growth factors, phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate, and retinoic acid, and is apparently essential for monocytic differentiation in HL-60 cells (54). H4TF-1 is a 105–110-kDa transcriptional activator that binds to a GGGGGAGGG consensus sequence in the human histone H4 gene (56, 57), is histone H4 specific, and is different from Sp1. UBP-1 is a 61–63-kDa protein that binds to the HIV *tar* element and TATA box, plays a role in tat-induced activation of the HIV-LTR, and recognizes a CTCTCTGG consensus site (59, 60). The CTCF factor is a 130-kDa protein that binds to a CCCTC motif with three repeats every 12–13 bp in the chicken c-*myc* promoter and likely functions as a negative regulator of cell growth and differentiation (58); however, only one such motif is present in the EPO promoter sequence. The EPO promoter also contains two putative PuF sites in functionally active/footprinted regions. PuF (also known as nm23-H2) is a 17-kDa human c-*myc* transcriptional activator originally isolated from HeLa cells that binds to a GGGTGGG consensus sequence (52). PuF is a nucleoside diphosphate kinase and a putative negative regulator of tumor metastasis (53). Finally, the EPO promoter contains a consensus site for GaEII (GGGGGGTT), a factor shown to interact with this G_6 motif in the adenovirus-2 EII-late promoter (61). Whether any of these factors play a role in EPO gene expression or regulation remains to be determined. We are currently investigating which if any of these factors are expressed in HL-60-C15 cells or during normal eosinophil differentiation, and/or bind to the functional elements thus far defined in the EPO promoter.

We recently described the expression of the family of GATA-binding proteins, including GATA-1, GATA-2, and GATA-3, in peripheral blood eosinophils obtained from patients with marked eosinophilia due to the hypereosinophilic syndrome, and in eosinophil-inducible HL-60 cell lines including the HL-60-C15 line used in the present studies (51). GATA-binding proteins had previously been shown to be important regulators of hematopoietic-specific transcription in the erythroid, megakaryotcyte, mast cell, and T cell lineages (76). However, the specific functional role of the GATA-binding proteins in the eosinophil lineage remains unknown. The eosinophil-inducible HL-60-C15 cell line was shown to constitutively express mRNA for all three GATA-binding proteins with expression of GATA-1 and GATA-3, but not GATA-2, strongly up-regulated by butyrate induction (51). In this regard, the 5′ putative promoter regions of three of the eosinophil granule cationic protein genes encoding MBP (13), ECP (15),[4] EDN (15), and the promoter for CLC protein (eosinophil lysophospholipase) (37) contain potential target GATA-binding sites,[4] whereas the promoter for EPO described in the present study does not. However, functional analyses are required to determine whether any of these potential GATA-binding sites are active in the eosinophil- or myeloid-specific regulation of these genes.

Hamann and colleagues (15) have performed alignments of the 5′-flanking sequences of EPO and MBP with those of EDN/ECP and reported similarity indices of ~54 and 51%, respectively. Similarities around several nucleotide blocks in these sequences, including heptamers that resemble an octamer-like motif (ATGCAAAT) found in immunoglobulin promoters and the immunoglobulin heavy chain enhancer, were identified (15), albeit in regions of the EPO promoter that appear to have no functional activity in the present study. Comparison of the promoter sequence of the EPO gene with that of the CLC promoter (37), or the 5′-flanking regions of MBP (13), ECP (15),[4] and EDN (15) show limited sequence similarity within the functionally active cis-elements and footprinted regions of the EPO promoter. A consensus site for CTCF (58) was also noted in the functional region of the CLC promoter (bp −159 to −163) (37), as well as in the 5′-flanking regions of MBP (13) and EDN (15), but is absent in the highly similar ECP upstream sequence (15).[4] One additional sequence (CCCCACCC), present at bp −43 to −50 of the EPO promoter overlapping the putative

[4] S. J. Ackerman and D. G. Tenen, unpublished observations.

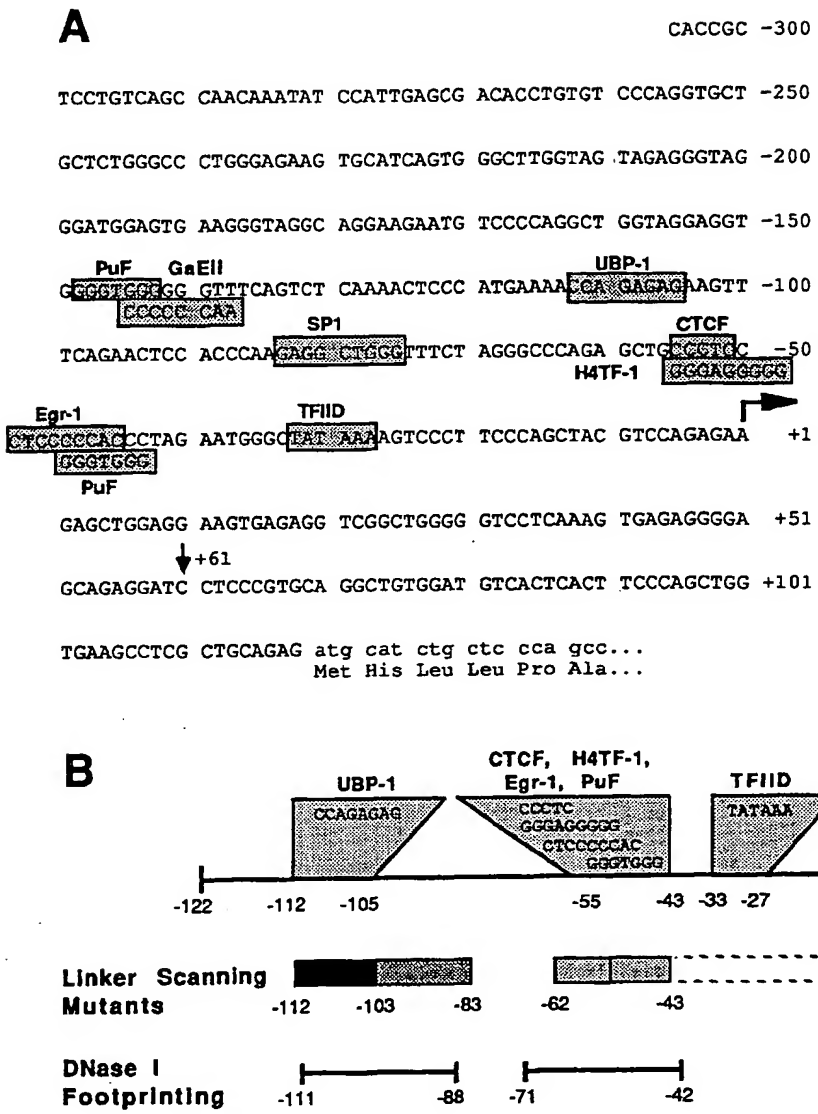


FIG. 9. **Functional cis-elements and potential trans-acting factors regulating the EPO promoter.** *Panel A*, the EPO promoter sequence (20) on the coding strand is shown from −306 bp through +137; bp +1 to +119 represents the 5′-untranslated region of the EPO mRNA and bp +120 to +137 (*lower case letters*) encodes the first 6 amino acids (21) (shown in *three-letter code* below the DNA sequence). The *arrow* (at +61) indicates the first bp of the 5′-untranslated region included in the EPO-pXP2 luciferase constructs analyzed in Figs. 5–8. The putative TATA box TFIID-binding site is *boxed*. Consensus sequences for potential DNA-binding proteins are *boxed* and *shaded* and include the PuF, Egr-1, H4TF-1, CTCF, UBP-1, and GaEII transcription factors (52–61). *Panel B*, comparison of the DNase I protected and functionally active regions of the EPO promoter. *Rectangular boxes* indicate the negatively (*black*) and positively (*shaded*) acting sequence elements which correspond to the DNase I-protected regions (*brackets*) and consensus sequences (*panel A*) for putative trans-acting factors for this promoter.

PuF and Egr-1 sites (Fig. 9), has been identified within the functional CLC promoter (bp −179 to −186) (37), as well as in other myeloid-specific genes including myeloperoxidase (33), cathepsin G (77), and neutrophil elastase (78). Finally, the murine myeloperoxidase promoter was recently characterized and shown to contain several functional elements, one of which binds a cell type restricted transcription factor MyNF1 (66); MyNF1 was recently identified as PEBP2αB and is the murine homolog of AML1 (79). Examination of the functional elements we have identified in the EPO promoter did not reveal any consensus sites for the MyNF1 transcription factor. In addition, alignment of the EPO 5′ upstream sequence with that of human myeloperoxidase did not show any significant regions of similarity within the functional cis-elements defined in the present analyses.

Study of the EPO promoter and nuclear factors regulating its expression should elucidate unique transcriptional features of eosinophil gene regulation in particular and myeloid gene expression in general in the differentiation of the various granulocyte lineages.

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